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(54) Title: METHOD AND DEVICE FOR DETECTING AND QUANTIFYING SUBSTANCES IN BODY FLUIDS

(57) Abstract

An *in vivo* method and apparatus for detecting an analyte in an individual. A sensor that includes a fluorescence reagent is placed in communication with the body fluids of the individual suspected of containing the analyte in such a way that once in place said sensor does not exit the skin of the individual. The sensor is configured to retain the fluorescence reagent while allowing analyte to diffuse into and out of said sensor. The sensor is illuminated transdermally, and the fluorescence from the fluorescence reagent associated with the presence of the analyte is measured.

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METHOD AND DEVICE FOR DETECTING AND QUANTIFYING
SUBSTANCES IN BODY FLUIDS
Background of the Invention

5   This application relates to detecting and
quantifying substances in body fluids using fluorescence
techniques.

Various methods for detecting and quantifying
substances in body fluids are known. For example, in the
10 case of glucose these methods include various
colorimetric reactions, measuring a spectrophotometric
change in the property of any number of products in a
glycolytic cascade or measuring the oxidation of glucose
using a polarographic glucose sensor.

15   U.S. Patent No. 4,401,122 discloses an in vivo
method for measuring substances such as glucose which
involves placing an enzyme (e.g., glucose oxidase) either
in or under the skin and detecting the enzymatic reaction
product (e.g., oxygen) directly through the skin either
20 colorimetrically or by using polarographic or enzyme
electrodes. The amount of enzymatic reaction product
detected is a measure of the amount of substrate.

Cerami, U.S. Patent No. 4,330,299 describes an
assay for measuring glucose concentration in which
25 detection is carried out by means of color-producing
agents or reagents which form precipitates. In one
embodiment, for example, rhodamine is bonded to a lectin
and a carbohydrate is affixed to a solid support. The
rhodamine-labelled lectin reversibly binds to the
30 carbohydrate in the absence of glucose. When glucose is
introduced, it binds to the lectin, thereby displacing
the rhodamine-labelled lectin from the carbohydrate. The
complexed and uncomplexed rhodamine-lectin are then
physically separated, and the reduction in color
35 intensity displayed by rhodamine-lectin bound to

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carbohydrate is measured as an indication of glucose
concentration.

Although such conventional assays have proven
reliable, the reagents on which they rely become
5 exhausted or must be removed. Therefore, these assays
require the use of disposable sticks or replaceable
cartridges, which can be expensive and inconvenient for
the active user.

Meadows and Schultz describe another method by
10 which blood glucose levels can be determined using
optical means. They describe a fiber optic glucose
sensor based on the competitive binding of glucose and
fluorescein-labelled dextran (FITC-dextran) to rhodamine-
labelled concanavalin A (Rh-Con A), Meadows, D. and J. S.
15 Schultz, Talanta, 35:145-150 (1988).

The Meadows and Schultz optical sensor is attended
by many problems, which means it is of limited use in a
clinical setting or in monitoring blood glucose levels in
individuals on a day to day basis. First, as mentioned
20 in the article, the sensor can only detect glucose
concentrations up to 2.00 mgs/ml. Although the normal
blood glucose concentration in man is approximately 1.00
mg/ml., the concentration of glucose in diabetic blood
can often exceed 3.00-4.00 mg/ml., which is well beyond
25 the upper limit of the sensor described.

Second, Meadow's and Schultz's sensor has a short
life because, as mentioned in the article, the dextran
aggregates and becomes precipitated. Third, only 45% of
the fluorescence is quenched using the Meadows and
30 Schultz optical sensor. This effect may not be dramatic
enough to be detected.

Finally, the in vivo use of a fiber optic is
clinically impractical because in order to work, it must
pierce the skin. Therefore, it requires an invasive
35 technique and puts the patient at significant risk for

developing serious infection. This is particularly true
in diabetic patients who are known to have reduced
resistance to infection.

5   An ideal sensor should be capable of detecting a
wide range of physiological concentrations of analyte.
As used herein, "physiological concentration" refers to
the concentration of analyte found in both normal and
pathological states. For example, in the case of glucose
it refers to glucose levels found in normal,
10   hypoglycemic, and hyperglycemic patients.

The sensor should also be reliable, reusable and
easy to use. In addition, the in vivo sensor should be
non-invasive.

Summary of the Invention

15   In a first aspect, the invention features an in
vivo method and sensor for detecting an analyte in an
individual (as used herein, "detecting" may include
qualitatively determining the presence of an analyte, as
well as quantitatively measuring its concentration). The
20   sensor is placed in communication with the body fluids
(e.g., blood, plasma, or serum) the individual suspected
of containing the analyte in such a way that once in
place the sensor does not exit the skin of the
individual. We refer to such placement as permitting
25   non-invasive detection and monitoring of the analyte.
The sensor includes a fluorescence reagent for detecting
the analyte and is configured to retain the fluorescence
reagent while allowing analyte to diffuse into and out of
the sensor. In this way, the sensor can be used for
30   extended monitoring because depletion of the fluorescence
reagent is minimized, if not eliminated. Once the sensor
is in place, it is illuminated with radiation
transdermally and the fluorescence from the fluorescence
reagent associated with the presence of the analyte is
35   measured.

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        As used herein, "fluorescence" refers to radiation
emitted in response to excitation by radiation of a
particular wavelength. It includes both short-lived
(nanosecond range) and long-lived excited state
5 lifetimes; the latter is sometimes referred to as
phosphorescence.

        As used herein, "fluorescence reagent" refers to a
material whose fluorescence behavior (e.g., intensity,
emission spectrum, or excitation spectrum) changes in the
10 presence of the analyte being detected.

        In some embodiments, it binds reversibly to the
analyte. For example, the reagent may be a fluorophore
(or compound labelled with such a molecule) that binds
directly to the analyte. It is the fluorescence behavior
15 of this molecule (or compound labelled with this
molecule) which changes as a result of analyte binding.

        The reagent may also include more than one
component. For example, it may include an analogue to
the analyte labelled with a fluorophore and a ligand
20 (e.g., an antibody, receptor for the analyte, lectin,
enzyme, or lipoprotein) that binds competitively (and
specifically) to the analogue and the analyte. In this
case, it is the fluorescence behavior of the labelled
analogue which changes as a result of ligand binding to
25 analyte. Conversely, ligand may be labelled, rather than
the analogue, in which case it is the fluorescence
behavior of the labelled ligand which changes.

        The reagent may also include two components, one
of which is labelled with an energy-absorbing donor
30 molecule and the other of which is labelled with an
energy-absorbing acceptor molecule; the donor and
acceptor have overlapping excited state energy levels.
One or both molecules forming the donor-acceptor pair can
be fluorophores. Regardless, however, it is the
35 fluorescence associated with the non-radiative resonance

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energy transfer from donor to acceptor that is measured.
This transfer is often referred to as "FRET" (an acronym
for non-radiative fluorescence resonance energy
transfer). The components may be members of a specific

5 binding pair (e.g., an analogue of the analyte and a
ligand capable of binding competitively (and
specifically) to both the analogue and the analyte) or
ligands (e.g., antibodies or oligonucleotides) that bind
specifically to different portions of the analyte.

10   FRET can also be measured where a single reagent
capable of binding to the analyte is labelled with both
donor and acceptor molecules.

          As used herein, "fluorophore" refers to a molecule
that absorbs energy and then emits light.

15   As used herein, "analogue" refers to a material
that has at least some binding properties in common with
those of the analyte such that there are ligands that
bind to both. The analogue and the analyte, however, do
not bind to each other. The analogue may be a derivative

20 of the analyte such as a compound prepared by introducing
functional chemical groups onto the analyte which do not
affect at least some of the binding properties of the
analyte. Another example of a derivative is a lower
molecular weight version of the analyte which nonetheless

25 retains at least some of the binding properties of the
analyte.

          In other embodiments, the fluorescence reagent
does not bind to the analyte except transiently. In
other words, binding to analyte in and of itself is not

30 the cause of the detectable fluorescence change.
Instead, in these embodiments detection involves an
irreversible chemical reaction, with an accompanying
change in fluorescence; the chemical reaction may be
proceeded by binding to analyte.

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In one example, the fluorescence reagent may
include an enzyme and a co-factor or substrate that
reacts with the analyte in the presence of the enzyme
with an accompanying fluorescence change. In the case
5 where the enzyme reacts with substrate, a co-factor may
also be present to assist the reaction.

In another example, the analyte is an enzyme and
the fluorescence reagent includes a substrate that is
altered (e.g., cleaved) by the enzyme with an
10 accompanying fluorescence change. In yet another
example, the fluorescence reagent includes (a) an enzyme
in whose presence the analyte reacts to form a detectable
product and (b) an indicator for detecting the presence
of the product.

15   In another example, the fluorescence reagent
includes (a) a substrate or co-factor capable of reacting
with an enzyme to cause a detectable fluorescence change,
(b) a first ligand labelled with a first portion of that
enzyme and capable of binding to a first portion of the
20 analyte, and (c) a second ligand labelled with a second
(different) portion of the enzyme and capable of binding
to a second (different) portion of the analyte. Neither
the first portion nor the second portion of the enzyme is
capable individually of interacting with the substrate or
25 co-factor to produce a detectable fluorescence change.
However, binding of the first and second ligands to the
analyte causes the first and second enzyme portions to
interact with each other, thereby reconstituting the
enzyme. The reconstituted enzyme can then react with the
30 substrate or co-factor, with a concomitant change in
fluorescence.

A variation of this approach involves an analogue
of the analyte labelled with a first portion of the
enzyme and a ligand labelled with a second portion of the
35 enzyme. In the absence of analyte, the analogue and

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analyte bind to each other, thereby reconstituting the
enzyme and allowing reaction with substrate or co-factor,
with accompanying fluorescence. The presence of analyte
disrupts analogue-ligand binding, and thus reconstitution
5 of the enzyme, with an accompanying decrease in
fluorescence (since the individual enzyme portions cannot
interact with the substrate or co-factor to cause a
detectable fluorescence change).

  Regardless of whether the fluorescence reagent
10 binds to analyte, changes in fluorescence associated with
the presence of the analyte may be measured in several
ways. These changes include changes in the excited state
lifetime of, or fluorescence intensity emitted by, the
fluorophore (or compound labelled with the fluorophore).
15 They also include changes in the excitation or emission
spectrum of the fluorophore (or compound labelled with
the fluorophore). Changes in the excitation or emission
spectrum, in turn, may be measured by measuring (a) the
appearance or disappearance of emission peaks, (b) the
20 ratio of the signal observed at two or more emission
wavelengths, (c) the appearance or disappearance of
excitation peaks, or (d) the ratio of the signal observed
at two or more excitation wavelengths.

  In the case of FRET, the illumination wavelength
25 may be selected such that it predominantly excites only
the donor molecule. We say "predominantly" because due
to bleed-through phenomena, it is possible that there
will be some acceptor excitation as well. Thus, as used
herein, "excitation" of donor or acceptor will refer to
30 an excitation wavelength that predominantly excites donor
or acceptor.

  Following excitation, non-radiative fluorescence
resonance energy transfer is determined by measuring the
ratio of the fluorescence signal at two emission
35 wavelengths, one of which is due to donor emission and

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the other of which is due to acceptor emission. Just as
in the case of excitation, there may be some "bleeding"
of the fluorescence signal such that acceptor emission
makes a minor contribution to the donor emission signal,
5   and vice versa. Thus, whenever we refer to a signal as
being "due to" donor emission or acceptor emission, we
mean that the signal is predominantly due to donor
emission or acceptor emission.

        Alternatively, the illumination may be selected
10   such that it excites the donor at a first wavelength and
the acceptor at a second wavelength. In other words, two
separate excitation events, each at a different
wavelength, are used. In this case, non-radiative
fluorescence energy transfer is determined by measuring
15   the ratio of the fluorescence signal due to the acceptor
following donor excitation and the fluorescence signal
due to the acceptor following acceptor excitation.

        FRET can also be measured by assessing whether
there is a decrease in donor lifetime, a quenching of
20   donor fluorescence intensity, or an enhancement of
acceptor fluorescence intensity; the latter two are
measured at a wavelength in response to excitation at a
different wavelength (as opposed to the ratio
measurements described above, which involve either
25   measuring the ratio of emissions at two separate
wavelengths or measuring the ratio of emission at a
wavelength due to excitation at two separate
wavelengths).

        A large number of analytes may be detected
30   according to the method of the invention. Suitable
analytes include, for example, carbohydrates (e.g.,
glucose, fructose, and derivatives thereof). As used
herein, "carbohydrate" refers to any of the group of
organic compounds composed of carbon, hydrogen, and
35   oxygen, including sugars, starches, and celluloses.

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Other suitable analytes include glycoproteins (e.g., glycohemoglobin, thyroglobulin, glycosylated albumin, and glycosylated apolipoprotein), glycopeptides, and glycolipids (e.g., sphingomyelin and the ganglioside G_{M2}).

5   Another group of suitable analytes includes ions. These ions may be inorganic or organic. Examples include calcium, sodium, chlorine, magnesium, potassium, bicarbonate, phosphate, and carbonate. The invention is also useful for detecting proteins and peptides (the

10 latter being lower molecular weight versions of the former); a number of physiological states are known to alter the level of expression of proteins in blood and other body fluids. Included within this group are enzymes (e.g., enzymes associated with cellular death

15 such as LDH, SGOT, SGTT, and acid and alkaline phosphatases), hormones (e.g., hormones associated with ovulation such as luteinizing hormone and follicle stimulating hormone, or hormones associated with pregnancy such as human chorionic gonadotropin),

20 lipoproteins (e.g., high density, low density, and very low density lipoprotein), and antibodies (e.g., antibodies to autoimmune diseases such as AIDS, myasthenia gravis, and lupus). Antigens and haptens are also suitable analytes.

25   Additionally, the invention is useful for detecting and monitoring analytes such as steroids (e.g., cholesterol, estrogen, and derivatives thereof). In the case of estrogen, the invention makes it possible to monitor menopausal patients under estrogen therapy (where

30 estrogen levels can be quite high). The invention is also useful for detecting and monitoring substances such as theophylline (in asthma patients) and creatinine (a substance associated with renal failure).

      The invention may also be used to detect and

35 monitor pesticides and drugs. As used herein, "drug"

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refers to a material which, when ingested, inhaled,
absorbed, or otherwise incorporated into the body
produces a physiological response. Included within this
group are alcohol, therapeutic drugs (e.g.,
5 chemotherapeutic agents such as cyclophosphamide,
doxorubicin, vincristine, etoposide, cisplatin, and
carboplatin), narcotics (e.g., cocaine and heroin), and
psychoactive drugs (e.g., LSD).

    The invention may also be used to detect and
10 monitor polynucleotides (e.g., DNA and RNA). For
example, overall DNA levels may be assayed as a measure
of cell lysis. Alternatively, the invention could be
used to assay for expression of specific sequences (e.g.,
HIV RNA).

15   To permit _in_ _vivo_ operation, the sensor may be
provided with a selectively permeable membrane that
permits analyte (but not fluorescence reagent) to diffuse
into and out of the sensor. In another embodiment, at
least some of the components of the fluorescence reagent
20 are immobilized within the sensor (e.g., on a substrate
or within the pores of a porous matrix). For example, in
the case of an analogue labelled with donor and a ligand
labelled with acceptor, one (or both) materials can be
immobilized. In another embodiment, at least some of the
25 components of the fluorescence reagent are freely mobile
(i.e., not immobilized) within the sensor.

    A drug delivery system in communication with the
sensor (e.g., via a biofeedback loop) may also be
implanted in the individual. The drug delivery system is
30 configured to release a prescribed amount of a drug in
response to the level of analyte detected by said sensor.

    In a second aspect, the invention features an _in_
vivo method for detecting an analyte in an individual
that is not limited to non-invasive monitoring.
35 According to this method, the sensor (containing a

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fluorescence reagent for detecting the analyte that
reversibly binds to the analyte is placed in
communication with the body fluids of the individual
suspected of containing the analyte. The sensor is
5 configured to retain the fluorescence reagent while
allowing analyte to diffuse into and out of said sensor.
The fluorescence reagent includes a specific binding
pair, one member of which is labelled with an energy-
absorbing donor molecule (which may be a fluorophore) and
10 the other of which is labelled with an energy-absorbing
acceptor molecule (which may be a fluorophore). The
excited state energy level of the donor overlaps with the
excited state energy level of the acceptor. The sensor
is illuminated so as to i) excite the donor or ii) excite
15 both the donor and acceptor. The fluorescence from the
fluorescence reagent associated with the presence of the
analyte is then measured by determining the extent to
which non-radiative fluorescence resonance energy
transfer ("FRET") occurs between the donor and the
20 acceptor upon binding. The non-radiative fluorescence
resonance energy transfer, in turn, is determined by
measuring i) the ratio of the fluorescence signal at two
emission wavelengths, one of which is due to donor
emission and the other of which is due to acceptor
25 emission, when only the donor is excited, or ii) the
ratio of the fluorescence signal due to the acceptor
following donor excitation and the fluorescence signal
due to the acceptor following acceptor excitation.

The method and devices of the present invention
30 can be used to detect a wide range of physiological
analyte concentrations (e.g., concentrations ranging from
0.5 to 18 mg/ml in the case of glucose). In addition,
the method is reliable. Also, because the reactants are
not consumed, the devices are reusable for extended

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periods. Moreover, many of the _in vivo_ embodiments are
non-invasive.

Other features and advantages of the invention
will be apparent from the following description of the
5 preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Figure 1A is a graphic representation of
absorbance and emission spectra of donor and acceptor
molecules.

10   Figure 1B is a representation of non-radiative
energy transfer.

Figure 2 is a graphic representation of the use of
FRET to measure glucose concentrations in a sample.

Figure 3 is a graph representing the effect that
15 increasing concentrations of glucose have on FRET between
the fluorescently labelled ligand, Rhodamine-ConA (RC)
and the fluorescently labelled glycoconjugate,
fluorescein BSA-Glucose (FBG).

Figure 4 is a graph representing FRET between FBG
20 and RC dialyzed against various concentrations of glucose
in Hanks Buffered Salt Solution (HBSS).

Figure 5 is a bar graph of Fl_{520}/Fl_{600} ratio for a
sample of FBG and RC dialyzed sequentially against
various different glucose concentrations in the
25 following: (reading left to right on the X axis) (0)
Hanks Buffered Salt Solution; (HS) normal horse serum;
(+5) horse serum + 5 mM glucose; (HS) horse serum; (HO)
normal horse serum + 10 mM glucose; (HS) normal horse
serum; (0) Hanks Balanced Salt Solution; (+10) normal
30 horse serum + 10 mM glucose; (HS) horse serum; (0) Hanks
Balanced Salt Solution.

Figure 6A is a graph representing FRET between FBG
and RC with no glucose present (control).

Figure 6B is a graph representing FRET between FBG
35 and RC microdialyzed against blood containing 3.2 mg/ml.

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of glucose, similar to what might be found in diabetic
patients.

   Figure 7 is a graph representing FRET in response
to 150 uls of a mixture of RC and FBG microdialyzed
5 against blood containing varying concentrations of
glucose.

   Figure 8 is a series of titration curves for
samples containing various glucose concentrations
measured over an eight week period.

10   Figure 9 is a graph representing FRET in response
to a mixture of RC and FBG microdialyzed against pooled
human serum containing various glucose concentrations.

   Figure 10 is an emission spectrum for a sensor
according to the invention implanted in a mouse.

15   Figure 11 is a photograph of a mouse provided with
an implant for sensing glucose underneath the skin of its
abdomen.

   Figure 12 is a schematic representation of a
fluorescently labelled ligand binding to analyte.

20   Figure 13 is a schematic representation of
fluorescently labelled donor and acceptor ligands which
bind to different portions of an analyte.

   Description of the Preferred Embodiments
A. Dual-Label Techniques
25   This group includes detection by means of FRET.
We will first describe the basic elements of FRET.
Basic Elements of FRET
   FRET generally involves the non-radiative transfer
of energy between two fluorophores, one an energy donor
30 (D) and the other an energy acceptor (A). Any
appropriately selected donor-acceptor pair can be used,
provided that the emission of the donor overlaps with the
excitation spectra of the acceptor and both members can

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absorb light energy at one wavelength and emit light
energy of a different wavelength.

The method is described below with particular
reference to fluorescein and rhodamine as the donor-
5 acceptor pair. As used herein, the term fluorescein
refers to a class of compounds which includes a variety
of related compounds and their derivatives. Similarly,
as used herein, the term rhodamine refers to a class of
compounds which includes a variety of related compounds
10 and their derivatives. Other examples of donor/acceptor
pairs are NBD N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) to
rhodamine, NBD or fluorescein to eosin or erythrosin,
dansyl to rhodamine, acridine orange to rhodamine.

Alternatively, both the donor and acceptor can
15 absorb light energy, but only one of them emits light
energy. For example, the donor can be fluorescent and
the acceptor can be nonfluorescent, or vice versa. It is
also possible to make use of a donor-acceptor pair in
which the acceptor is not normally excited at the
20 wavelength used to excite the (fluorescent) donor;
however, nonradiative FRET causes acceptor excitation.

Although the donor and the acceptor are referred
to herein as a "pair", the two "members" of the pair can,
in fact, be the same substance. Generally, the two
25 members will be different (e.g., fluorescein and
rhodamine). It is possible for one molecule (e.g.,
fluorescein, rhodamine) to serve as both donor and
acceptor; in this case, energy transfer is determined by
measuring depolarization of fluorescence.
30   The concept of FRET is represented in Figure 1.
The absorbance and emission of donor, designated A(D),
and E(D), respectively, and the absorbance and emission
of acceptor, designated A(A) and E(A), respectively, are
represented graphically in Figure 1A. The area of
35 overlap between the donor emission and the acceptor

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absorbance spectra (which is the overlap integral) is of
importance.   If excitation occurs at wavelength I, light
will be emitted at wavelength II by the donor, but not at
wavelength III by the acceptor because the acceptor does
5 not absorb light at wavelength I.

The non-radiative transfer process which occurs is
represented in Figure 1B.   D molecule absorbs the photon
whose electric field vector is represented by E.   The
excited state of D is shown as a dipole with positive
10 charge on one side and negative charge on the other.   If
an acceptor molecule (A) is sufficiently close to D
(e.g., typically less than 100 Angstroms), an oppositely
charged dipole is induced on it (it is raised to an
excited state).   This dipole-induced dipole interaction
15 falls off inversely as the sixth power of donor-acceptor
intermolecular distance.

Classically, partial energy transfer can occur.
However, this is not what occurs in FRET, which is an all
or nothing quantum mechanical event.   That is, a donor is
20 not able to give part of its energy to an acceptor.   All
of the energy must be transferred and energy transfer can
occur only if the energy levels (i.e., the spectra)
overlap.   When A leaves its excited state, the emitted
light is rotated or depolarized with respect to the
25 incident light.   As a result, FRET manifests itself as a
decrease in fluorescence intensity (i.e., decrease in
donor emission) at II, an appearance of fluorescence
intensity at III (i.e., an increase in sensitized
emission) and a depolarization of the fluorescence
30 relative to the incident light.

A final manifestation of FRET is in the excited
state lifetime.   Fluorescence can be seen as an
equilibrium process, in which the length of time a
molecule remains in its excited state is a result of
35 competition between the rate at which it is being driven

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into this state by the incident light and the sum of the
rates driving it out of this state (fluorescence and non-
radiative processes). If a further nonradiative process,
FRET, is added (leaving all else unchanged), decay is
5 favored, which means donor lifetime at II is shortened.
      When two fluorophores whose excitation and
emission spectra overlap are in sufficiently close
proximity, the excited state energy of the donor molecule
is transferred by a resonance dipole-induced dipole
10 interaction to the neighboring acceptor fluorophore. In
FRET, a sample or mixture is illuminated at a wavelength
which excites the donor but not the acceptor molecule
directly. The sample is then monitored at two
wavelengths: that of donor emissions and that of
15 acceptor emissions. If donor and acceptor are not in
sufficiently close proximity, FRET does not occur and
emissions occur only at the donor wavelength. If donor
and acceptor are in sufficiently close proximity, FRET
occurs. The results of this interaction are a decrease
20 in donor lifetime, a quenching of donor fluorescence, an
enhancement of acceptor fluorescence intensity, and
depolarization of fluorescence intensity. The efficiency
of energy transfer, E_t, falls off rapidly as the distance
between donor and acceptor molecule, R, increases. For
25 an isolated donor acceptor pair, the efficiency of energy
transfer is expressed as:

$$E_t = 1/[1 + (R/R_o)^6] \qquad (1)$$

where R is the separation distance between donor and
acceptor and R_o is the distance for half transfer. R_o is
30 a value that depends upon the overlap integral of the
donor emission spectrum and the acceptor excitation
spectrum, the index of refraction, the quantum yield of
the donor, and the orientation of the donor emission and
the acceptor absorbance moments. Forster, T., $\underline{Z}$

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Naturforsch. 4A, 321-327 (1949); Forster, T., Disc. Faraday So. 27, 7-17 (1959).

Because of its $1/R^6$ dependence, FRET is extremely dependent on molecular distances and has been dubbed "the
5   spectroscopic ruler". (Stryer, L., and Haugland, R. P., Proc. Natl. Acad. Sci. USA, 98:719 (1967). For example, the technique has been useful in determining the distances between donors and acceptors for both intrinsic and extrinsic fluorophores in a variety of polymers
10   including proteins and nucleic acids. Cardullo et al. demonstrated that the hybridization of two oligodeoxynucleotides could be monitored using FRET (Cardullo, R., et al., Proc. Natl. Acad. Sci., 85:8790-8794 (1988)).

15   Concept of Using FRET for Analyte Detection

In general, FRET is used for analyte detection is one of two ways. The first is a competitive assay in which an analogue to the analyte being detected and a ligand capable of binding to both analogue and analyte
20   are labelled, one with a donor fluorophore and the other with an acceptor fluorophore. Thus, the analogue may be labelled with donor and the ligand with acceptor, or the analogue may be labelled with acceptor and the ligand with donor. When the labelled reagents contact analyte,
25   analyte displaces analogue bound to ligand. Because ligand and analogue are no longer close enough to each other for FRET to occur, the fluorescence signal due to FRET decreases; the decrease correlates with the concentration of analyte (the correlation can be
30   established in a prior calibration step). In order to be able to reuse the fluorescence reagents, the binding between analyte and ligand should be reversible under physiological conditions. Similarly, the equilibrium binding constants associated with analyte-
35   ligand binding and analogue-ligand binding should be such

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that analyte can displace analogue. In other words,
analogue-ligand binding should not be so strong that
analyte cannot displace analogue.

     This approach is applicable to detection of
 5 carbohydrates, steroids, proteins, peptides, steroids,
antigens, haptens, drugs, pesticides, theophylline,
creatinin, and small organic molecules generally. In the
case of carbohydrates such as glucose and fructose,
suitable analogue-ligand combinations satisfying the
10 above-described selection criteria include the following
combinations: glycoconjugate-lectin, antibody-antigen,
receptor-ligand, and enzyme-substrate. For example, in
the case of glucose the combination of bovine serum
albumin covalently labelled with glucose or glucose
15 analogue (as the glycoconjugate) and Concanavalin A (as
the lectin) have been found to be effective. To
determine suitable combinations for other sugars, one can
select a lectin that binds to the sugar and then use that
lectin in combination with bovine serum albumin
20 covalently labelled with that sugar or an analogous
sugar.

     In the case of analytes such as steroids,
proteins, and peptides, the appropriate combination would
be an analogue to the steroid, protein, or peptide, and
25 an antibody (or antigen, where the protein or peptide is
an antibody) or a receptor for the steroid, protein, or
peptide. For example, in the case of steroids we refer
to Haugland, R.P. (1989) Molecular Probes: Handbook of
Fluorescent Probes and Research Chemicals, Molecular
30 Probes, Eugene, Oregon for preparation of suitable
analogues. Using cholesterol as a representative
example, derivatives can be prepared either by covalent
attachment of a fluorophore (e.g., NBD or pyrene) to the
aliphatic side chain or to a hydroxyl group (e.g., using
35 anthracene as the fluorophore). For cholesterol, the

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molecules thus produced are, respectively, 22-(N-(7-
nitrobenz-2-oxa-1,3 diazol-4-yl) amino-23,24-bisnor-5-
cholen-3B-ol; 1-pyrenemethyl 3B-hydroxy-22,23-bisnor-5-
cholenate; and cholesteryl anthracene-9-carboxylate. The
5 steroid can also be conjugated to a carrier protein or
other macromolecule which would also be fluorescently
tagged with donor or acceptor. The conjugation would
again proceed via either the aliphatic side chain or the
hydroxyl group.
10   Similar considerations apply in the case of
glycoproteins, glycopeptides, and glycolipids. In the
case of glycosylated hemoglobin, FRET between a labelled
lectin and the heme itself could be measured (this would
manifest itself as a quenching of fluorescence).
15   The second approach using FRET is to select two
ligands that bind to different portions (sites) of the
analyte molecule; in addition to being spatially
different, the portions may be chemically different as
well. This approach (illustrated schematically in Figure
20 13) is applicable to detection of antigens, haptens,
steroids, proteins, peptides, steroids, drugs,
pesticides, theophylline, creatinin, and large organic
molecules generally. The ligands could be two
antibodies, two cell receptors, or an antibody and a cell
25 receptor. For example, in the case of hormones such as
HCG, FSH, and LSH the labelled ligands could be
antibodies or cell receptors that bind to different
portions of the hormone molecule.
          One variation of this second approach is to detect
30 antibodies such as anti-DNA antibodies in lupus patients
by encapsulating two fluorescent DNA fragments, one
labelled with donor and the other with acceptor, and then
measuring FRET (which would occur if the antibody of
interest were present and crosslinked the labelled
35 fragments).

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Another variation involves labelled oligonucleotide probes. As described in Cardullo, R., et al., Proc. Natl. Acad. Sci., 85:8790-8794 (1988), the hybridization of two oligodeoxynucleotides can be
5 monitored using FRET in conjunction with such probes. In this way, specific DNA sequences can be determined.

To assay overall DNA levels, reagents that bind non-specifically to DNA or RNA are used. Examples of such reagents include fluorescent intercalating dyes
10 which show dramatic spectral shifts upon binding.

In yet another variation, a single material is labelled with both donor and acceptor fluorophores. The fluorescence change associated with the conformational change in the material upon binding to analyte is used as
15 an indication of analyte presence. For example, the analyte may be a helical DNA molecule and the fluorescence reagent is a material labelled with donor and acceptor fluorophores that binds to the DNA. Binding changes the separation distance between the donor and
20 acceptor, and thus the signal detected by FRET.

We now describe the use of FRET in a competitive binding assay format to determine glucose concentration.
<u>Concept of Using FRET to Measure Glucose Concentrations</u>

The concept of using FRET to measure glucose
25 concentrations in solution is represented in Figure 2. One macromolecule (designated M) includes a number of covalently-bound fluorophores and glucose residues and is referred to as a glycoconjugate. A second macromolecule (designated L) includes a ligand which has a high degree
30 of specificity for glucose (e.g., concanavalin A) and a fluorophore which is generally not the same fluorophore as that on the first macromolecule.

One of these fluorophores is chosen to be a donor and the other is an acceptor as described previously.
35 For the purposes of this illustration, the donor molecule

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has been placed on the glycoconjugate and the acceptor
has been placed on the ligand. The association can then
be diagrammed as:

                  DMG + AL----------→ DMG-LA,

5 where DMG stands for Donor-Macromolecule-Glucose, AL
stands for Acceptor-Ligand, and DMG-LA represents the
association between the glucose present in the first
complex and the ligand present in the second complex.
Upon association, the two macromolecules are now close

10 enough to allow energy transfer between the donor and the
acceptor to occur.

     The presence of free glucose introduces a
competitive inhibitor into the formula because free
glucose competes with the conjugated glucose for the

15 ligand. Thus, increasing concentrations of glucose
produces a decrease in the amount of ligand binding to
the glycoconjugate. At relatively low concentrations of
glucose, the transfer efficiency will remain high, since
little of the macromolecular association will be

20 affected. At high concentrations of glucose, the
transfer efficiency will be low, due to the fact that the
glucose has successfully competed the ligand off of the
complementary macromolecule.

     As described in the following sections, it has

25 been shown that it is possible to obtain a reliable,
repeatable measure of glucose in a sample containing
glucose concentrations within the range typically found
in normal individuals and in those in whom glucose
homeostasis is altered (e.g., in diabetic and

30 hypoglycemic patients). Further, it has been shown that
the reactants used (i.e., fluorescently-labelled ligand
and glycoconjugate) are stable and can be reused.

     Competition experiments in which FRET was measured
for various concentrations of glucose in Hanks Buffered

35 Salt Solution (HBSS) were conducted. These experiments

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are described in detail in Example 3. Spectra were
collected by exciting fluorescein at 472 nm and scanning
the emission from 500-650 nm. Typically, fluorescence
intensities were monitored at the emission maxima for
5 fluorescein (about 520 nm) and rhodamine (about 600 nm).
The measure of energy transfer in these studies was
either the ratio of fluorescence intensities at 520 nm
and 600 nm (i.e., FI 520/FI 600) as a function of glucose
concentration or the quenching of fluorescein at 520 nm.
10   A number of observations were made during these
trials that indicate that the method of the subject
invention provides a reliable means of detecting glucose
over a wide range (i.e., glucose concentrations found in
normal, hypoglycemic and diabetic subjects). Firstly,
15 the compounds were found to be stable. Figure 3 shows
that the compounds exhibited the same fluorescence
properties over a one week time period in response to
glucose concentration. Data in the graph reflect the
change in fluorescence intensity at 520 nm from 0 mM
20 glucose as a function of glucose concentration.
        Another experiment testing the stability of the
sensor is described in Example 4. In this experiment,
titration curves were periodically prepared over the
course of ten weeks for human serum samples containing
25 various known concentrations of glucose. Figure 8
demonstrates that the system continues to respond to
glucose for 10 weeks. The data in the graph reflect the
ratio of fluorescein emission (at 520 nm) to rhodamine
emission (at 600 nm).
30   Figure 4 shows that the FRET method was able to
predict glucose concentrations accurately at
concentrations up to 31 mM glucose (~600 mg/dL), which is
well within and in excess of the desired range. Normal
glucose concentrations in blood are usually between 80
35 and 120 mg/dL and diabetic levels can exceed 500 mg/dL.

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In the range of 0 to 31 mM glucose, the response by FRET
was nearly linear with a coefficient of determination
(r^2) of 0.983. Therefore, the sensor is reliable for
detecting glucose concentrations over the entire
5 physiological range (i.e., for normal and hyperglycemic
(diabetic) individuals).

The components of the present invention were also
found to be reusable. When FBG and RC were placed into
8-11 kD cutoff dialysis tubing and dialyzed against
10 different glucose concentrations in serum, FRET
accurately determined these concentrations in accordance
with solution studies. The initial volume of FBG and RC
in HBSS was 2 ml and dialyzed against different glucose
concentrations in 50 ml volumes. In general, it took
15 approximately 30 minutes to reach a plateau level in the
FI_{520}/FI_{600} ratio. As shown in Figure 5, the response is
reversible as the dialysis tubing was placed into
different glucose concentrations and then equilibrated.
Experiments in horse serum showed that the response was
20 not affected by dialyzed components, but did shift the
baseline due to light scattering.

Another experiment demonstrating reusability is
described in Example 5. The results, shown in Figure 9,
demonstrated that the sensor could be used repeatedly to
25 measure samples having various glucose concentrations
without significant changes in the response.

The ability of the present method to determine the
glucose concentration in hyperglycemic blood (i.e., a
sample of normal blood augmented with glucose to produce
30 glucose levels observed in diabetic individuals) was also
assessed. Figure 6 shows the raw data scans from this
experiment. The first (Figure 6A) is an emission scan
from 500 nm of a sample excited at 472 nm which does not
contain glucose. It shows considerable energy transfer,
35 as evidenced by the ratio of the two emission peaks. The

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rhodamine (second) peak is actually higher than the
fluorescein (first) peak.

    The second scan depicted in Figure 6B shows the
effect of dialyzing against a blood sample with
5 hyperglycemic levels of glucose, here 317 mg/dL. The
energy transfer decreases as seen both in the increase of
the fluorescein emission peak and a concurrent decrease
in the sensitized rhodamine emission.

    The present method was shown to be effective in
10 assessing glucose levels in blood and is sensitive in the
hyperglycemic range in the following way: 150 μm of a
mixture of Rh-Con A and FBG was microdialyzed for 15
minutes against 1500 μl samples of hyperglycemic blood.
Figure 7 shows that the initial fluorescein/rhodamine
15 fluorescence ratio was just above 1.6 and that the
fluorescein/rhodamine fluorescence ratio increased as a
function of glucose concentration, saturating at about 40
mM glucose.

    EXAMPLE 1   Preparation of FBG and RC

20   Fluorescein-BSA-Glucose (FBG) purchased from Sigma
and Rhodamine-ConA (RC) purchased from Molecular Probes
were dissolved in Hanks Buffered Salt Solution (HBSS)
from Gibco to a final concentration of 2 mg/ml. The
solutions were then centrifuged at 10,000 g for 30 min.
25 to remove large particulates. The supernatant was then
collected and placed on a 10,000 MW Amicon
ultrafiltration device and centrifuged at 2,000 g until
the ultra-filtrate had passed through the membrane. The
retained material was then resuspended in 2 mls. of HBSS
30 and this procedure was repeated until no free fluorescein
or rhodamine was detected in the ultrafiltrate.

    The final retained material was collected and
resuspended in 2 ml. of HBSS and spun one more time at

- 25 -

10,000 g for 30 min. The supernatant was collected and
stored at 4°C until used for energy transfer experiments.

EXAMPLE 2   Determination of the Optimal Concentrations of
Fluorescein-BSA Glucose and Rhodamine-ConA

5   Before competition experiments could be performed
with mannose and glucose, optimal concentrations of
Fluorescein-BSA-Glucose (FBG) and Rhodamine-Con A (RC)
had to be determined. In any solution study using energy
transfer, one must avoid the trivial possibility that

10 FRET is occurring simply because the molecules are close
enough to one another in solution. Thus, FRET solution
studies should ideally be done at concentrations less
than 1 mM because at that concentration the molecules are
separated by 120A units (far enough apart so they

15 specifically interact).

        Conversely, in these experiments, the
concentrations of fluorophore must be high enough to be
detected and the concentration of the two species (FBG
and RC) must be above their binding constants (K_bs).

20   Initial concentrations of FBG and RC were
approximately 2 mg/ml. Maximum transfer occurred when
final concentration of FBG was approximately 2 μg/ml and
the final concentration of RC was approximately 150
μg/ml. Optimization of FRET was determined by quenching

25 of fluorescein fluorescence, which was 50% maximal at 30
μg/ml.

EXAMPLE 3

Competition Experiments with Mannose and Glucose

        The spectrofluorimeter used for these studies was

30 a Perkin-Elmer MPF-2 equipped with a temperature stage
and interfaced to an Apple IIE computer.

        Concanavalin A, (Con A) is a lectin that
specifically binds glucose. A serial dilution of glucose
was performed using approximately 2 μg/ml FBG and

35 approximately 150 μg/ml RC from 0-5 mM glucose in HBSS.

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Various concentrations of glucose were the concentration
of glucose increases, the fluorescence intensity at 520
nm increases, and that the 1/2 maximal response for
glucose was approximately 1.87 mg/ml.

5 EXAMPLE 4 TITRATION CURVE STABILITY STUDIES

    Because the issue of reusability is related to the
issue of stability, an experiment was carried out to
demonstrate the chemical stability of the sensor. A
sterile mixture of FBG (0.6 μM) and RC (4.6 μM) in Hepes
10 Saline was prepared. From this mixture, 75 μL aliquots
were prepared and stored in the dark at room temperature
for up to eight weeks. Periodically, a set of aliquots
was taken to prepare a titration curve using human serum
samples (each representing a pool of donors) to which
15 various known concentrations of glucose had been added.
The curve was prepared by adding an aliquot of the FBG/RC
solution to each serum/glucose sample and then measuring
the F/R ratio for each sample. The results, graphically
in Figure 8, demonstrate that the sensor continues to
20 respond to glucose for 10 weeks.

EXAMPLE 5 REUSABILITY STUDY

    The following experiment was performed to
demonstrate that the same aliquot of RC-FBG mixture could
be used over and over again to measure glucose
25 concentration in various samples.

    A mixture of RC (2 μM) and FBG (1 μM) in Hepes
Saline was prepared. Approximately 30 μL of the mixture
was placed in a polyacrylonitrile ("PAN-69") hollow fiber
(inner diameter approx. 0.5 mm; length approx. 1 in.;
30 molecular weight cutoff 10 kDa). PAN fibers were chosen
because they are reasonably biocompatible and resist
clogging. The ends of the fiber were sealed and an
initial reading of the F/R ratio taken. The fiber was
then sequentially equilibrated by dialysis for 10 minutes
35 at room temperature against 200 μL samples of pooled

human serum to which various known concentrations of
glucose (measured using a Beckman glucose analyzer) had
been added, after which the F/R ratio of each sample was
measured. The fiber was then stored at 4°C in human

5 serum (55.6 mg/dL glucose) overnight and the samples re-
measured 24 hours later. The data from both sets of
readings is shown graphically in Figure 9. The results
from both days are essentially the same. Similar data
(not shown) was obtained when measurements were taken on

10 a third day. In addition, similar results were obtained
using polysulfone fibers.

The results of this experiment demonstrate that
the sensor can be used repeatedly to measure samples
having various glucose concentrations without significant

15 changes in the response.

B.   Single Label Techniques

These techniques generally fall into two
categories: (1) those involving direct binding of a
fluorophore or compound labelled with the fluorophore to

20 the analyte of interest, and (2) those involving a
competitive assay in which the analyte and an analogue
thereof compete for the same ligand (either the analogue
or ligand being labelled with a fluorophore. Regardless
of the category, however, fluorescence changes caused by

25 the presence of analyte are detected by measuring changes
in fluorescence intensity or in excitation or emission
spectra, as described in the Summary of the Invention,
above.

One embodiment of the single label technique is

30 illustrated schematically in Figure 12. In the case of a
competitive assay protocol, an analogue capable of
binding competitively to the ligand would also be
included.

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The direct binding protocol is particularly useful
in the detection and measurement of ions. Examples
include calcium, magnesium, sodium, chlorine, and
potassium ions. Fluorescent probes for these ions (as
5 well as other substances) are known and generally
described, e.g., in Haugland, R.P. (1989) Molecular
Probes: Handbook of Fluorescent Probes and Research
Chemicals, Molecular Probes, Eugene, Oregon. The probes
may be used alone or may be bonded to another compound,
10 e.g., a macromolecule such as dextran. In fact, some of
these probes are commercially available immobilized in a
matrix or bound to macromolecules such as dextran.

Other examples include probes sensitive to blood
pH (which involves detection of hydrogen ion
15 concentration). Suitable pH probes are described in
Haugland (above) and include SNARF, SNAFL, and BCECF.

In the case of calcium ions, many of the probes
are derivatives of ethylene diamine tetraacetic acid
(EDTA), a chelating agent that binds the calcium ion.
20 They include, for example, the dyes Fura-2, Indo-1, quin-
2, fluor-3, and rhod-2, all of which are probes for
calcium ions. In the case of quin-2, fluor-3, and rhod-
2, fluorescence changes are measured by measuring changes
in fluorescence intensity upon analyte binding. Fura-2
25 and Indo-1, on the other hand, undergo changes in
excitation or emission spectra upon analyte binding.

The competitive binding approach is useful for the
classes of analytes described above in the case of FRET
detection. Selection of appropriate analogue-ligand
30 combinations is as described therein, the only difference
being that only one material (i.e., either analogue or
ligand) is labelled with a fluorophore. Suitable
fluorophores are those which are environmentally
sensitive, i.e., probes whose fluorescence behavior
35 changes depending on the chemical environment in which

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they find themselves (the presence of analyte being an
example of an environmental change). Examples of
suitable fluorophores include (in addition to those set
out in the case of FRET) NBD, dansyl, pyrene, anthracene,
5 and indocarbocyanine fluorophores.

C.   Non-binding Technique

In this type of assay, the fluorescence reagent
does not bind to the analyte (i.e., binding in and of
itself does not cause the detectable fluorescence
10 change).

One example of such a sensor includes an enzyme
and a co-factor or substrate that reacts with the analyte
in the presence of the enzyme, with an accompanying
fluorescence change. For example, the sensor may be an
15 ethanol sensor in which NAD (the co-factor) and alcohol
dehydrogenase (the enzyme) are included in the sensor.
In the presence of ethanol, NAD is converted to NADH,
which is fluorescent. The change in fluorescence upon
production of NADH is used to detect the presence and
20 concentration of alcohol in the body.

Another example of such a sensor is a glucose
sensor that proceeds by way of two reactions: an initial
enzyme reaction that converts glucose into a detectable
product and an indicator reaction that detects the
25 product. In the first reaction, glucose reacts in the
presence of a first enzyme (glucose oxidase) to produce
hydrogen peroxide. In the second reaction, the presence
of hydrogen peroxide is detected by a dye (leuco dye) in
a reaction catalyzed by a second enzyme (peroxidase).
30 The change in fluorescence upon production of hydrogen
peroxide (as evidenced by the dye) indicates the presence
and concentration of glucose. The sensor thus includes
two enzymes (peroxidase and glucose oxidase) and a
fluorophore (e.g., leuco dye) sensitive to the reaction
35 product of the first reaction.

- 30 -

In another example of such a sensor, the analyte
is an enzyme. The sensor includes a substrate that is
cleaved by the enzyme to create by-products having
different fluorescence properties compared to the
5 uncleaved substrate. For example, the enzyme may be
trypsin and the substrate may be N-CBZ-Gly-Gly-Arg-beta-
naphthylamide HCL. Cleavage releases an N-CBZ-containing
fragment whose fluorescence properties are different from
the uncleaved substrate.
10   In another example of such a sensor, the sensor
includes a co-factor or substrate that reacts with an
enzyme to cause a detectable fluorescence change, and two
ligands that bind to different portions of the analyte.
Each ligand is labelled with a portion of the enzyme that
15 reacts with the substrate or co-factor. The two enzyme
portions are different from each other. Moreover,
individually they are incapable of interacting with the
substrate or co-factor to produce a detectable
fluorescence change. However, when the two labelled
20 ligands bind to analyte the individual enzyme portions
interact with each other to reconstitute the enzyme (the
enzyme portions are chosen such that reconstitution does
not occur, or occurs only to a minimal extent, until
ligand binding to analyte occurs). Once the enzyme is
25 reconstituted, it can now react with the substrate or co-
factor.
In a variation of this protocol, an analogue of
the analyte is labelled with one portion of the enzyme
and a ligand capable of binding to both analogue and
30 analyte is labelled with the other portion. When
analogue and ligand bind to each other, the enzyme is
reconstituted and reaction with substrate or co-factor
occurs. Once again, the enzyme portions are chosen such
that reconstitution doe not occur, or occurs only to a
35 minimal extent, until analogue-ligand binding occurs.

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The presence of analyte disrupts analogue-ligand binding, and thus enzyme reconstitution. As a result, fluorescence associated with reaction between enzyme and substrate or co-factor decreases, the decreases being

5 associated with analyte presence and concentration. Because reaction with substrate or co-factor occurs in the absence of analyte, the substrate/co-factor and enzyme should be chosen such that this reaction occurs slowly. This will minimize depletion of substrate/co-

10 factor, thereby enabling the sensor to be used over extended periods of time.

D.   In Vivo Use

          In vivo embodiments of this invention are directed to measurement of analytes by placing the fluorescence

15 reagent in communication with (e.g., contacting) body fluid containing the analyte of interest. The reactants comprising the fluorescence reagent can be placed in, on, or under the skin. Alternatively, the reactants can be placed within an organ or a vessel (e.g., a vein or

20 artery) in which they are in communication with the analyte, which can then be measured by the present method. In the embodiment in which the reactants are positioned in, on or under the skin, the analyte is detected by illuminating the skin transdermally. The

25 fluorescence signal is then detected transdermally using, e.g., a fluorimeter.

          A variety of modes of placing the reactants in communication with the analyte may be employed. The encapsulated sensor can be implanted anywhere in the

30 body. The reactants can be introduced into the body in any type of supporting or surrounding material which retains the reactants at the desired location and also allows contact or communication with the analyte such that it can be measured (e.g., its concentration can be

35 determined by the present method). The sensor could also

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be provided with a semipermeable membrane that allows
analyte to diffuse freely into and out of the sensor, but
not the fluorescence reagents. Selectivity can be based
upon molecular size or upon electrostatic or chemical
5 characteristics.

Some or all of the reactants comprising the
fluorescence reagent may be immobilized, e.g., on a
substrate or within the pores of a porous matrix. Such
an approach would be particularly useful in the case of
10 assays based upon a single reactant that interacts with
the analyte and causes a quenching/enhancement of
fluorescence or a change in emission or excitation
spectrum. However, immobilization techniques can be used
in the case of the multi-component competitive assay
15 format as well. For example, in the case of a
competitive assay involving a fluorescently labelled
analogue and a ligand, the analogue could be immobilized
on the substrate and the ligand attached to the analogue
via a long, flexible, linker arm.
20   In another procedure, reactants may be mixed with
an adjuvant, e.g., silicone or fluorocarbon oils, in
which the reactants freely diffuse (as opposed to being
immobilized) and interact with each other, and injected
as a bolus intra- or subcutaneously. The adjuvant must
25 exchange the analyte (but not the reactants) with the
surrounding body fluid, must allow all the reactants to
interact successfully, and must be immiscible (or only
slowly miscible) with water. This approach is
particularly useful in FRET-based assays because it
30 allows the labelled reactants to freely diffuse and
associate or dissociate with each other.

The reactants may be tattooed onto the skin. For
example, the reactants may be tattooed directly or
admixed with an adjuvant and then tattooed. They may

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also be encapsulated in microcapsules and the
microcapsules then tattooed onto the skin.

In another embodiment, the reagents may be
contained in a transcutaneous patch.

5   The reactants may also be modified in such a way
that when injected subcutaneously, they become bound to
cell structure and therefore remain fixed in situ under
the skin. For example, RC is known to bind to cells. In
addition, the albumin of the FBG complex can be

10 engineered to include a reactive group that binds cells.

In order to determine whether, as an example, an
in vivo glucometer is feasible, experiments were
conducted in which solutions of RC (150 μg/ml) and FBG (2
μg/ml) were injected into mouse skin. Illumination with

15 a laser at the appropriate wavelengths produced strong
signals from both the fluorescein and the rhodamine;
energy transfer occurred and was detected.

Any in vivo mode of placing the reactants in
communication with analyte can be modified to include a

20 drug delivery system. The drug delivery system therefore
inject drug into a patient upon detection of
inappropriately high analyte levels. One example would
be an insulin pump that injected insulin upon detection
of abnormally high glucose levels. Another would be a

25 pump that injected chemotherapeutic agent.

To demonstrate that a sensor according to the
invention will work in vivo, the following experiment was
performed. Although described here for glucose, it is
readily applicable to other analytes as well.

30 EXAMPLE 6

A Pan-69 fiber (approx. 0.75 inches long) was
loaded with RC (9 μM) and FBG (0.4 μM) as described above
in Example 5. The fur covering the abdomen of a diabetic
mouse was removed using a depilatory and the fiber

35 surgically implanted subcutaneously under the denuded

- 34 -

skin. The mouse was positioned in a fluorimeter so that
approximately 0.5 inch of the implanted fiber was
illuminated by a focused xenon lamp (472 nm) at what is
normally the center of the cuvette. Slits for both
5 monochromators were set at 8 nm. The emission signal was
scanned from 500 nm. The results are shown in Figure 10.

  This experiment demonstrates that the sensor can
be implanted and provide a detectable signal which
enables one to determine the 520/600 ratio as a measure
10 of energy transfer.

  Figure 11 is a photograph prepared from a low
light video of a mouse provided with an implant
underneath the skin of its abdomen as described above.
The mouse is lying on a microscope stage which is used as
15 a convenient light source. The implant is excited with
green light. The emitted red light is observed through a
red filter. The glowing line on the mouse's abdomen is
the fluorescence from the implant.

  Other embodiments are within the following claims.
20   What is claimed is:

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1.   An in vivo method for detecting an analyte in an individual comprising the steps of

a) placing a sensor in communication with the body fluids of said individual suspected of containing said
5 analyte in such a way that once in place said sensor does not exit the skin of the individual,

said sensor comprising a fluorescence reagent for detecting said analyte,

said sensor being configured to retain said
10 fluorescence reagent while allowing analyte to diffuse into and out of said sensor;

b) illuminating said sensor transdermally; and

c) measuring the fluorescence from said fluorescence reagent associated with the presence of said
15 analyte in said individual.

2.   The method of claim 1 wherein said fluorescence reagent reversibly binds to said analyte.

3.   The method of claim 1 wherein said analyte comprises a carbohydrate.

20   4.   The method of claim 3 wherein said carbohydrate comprises glucose or a derivative thereof.

5.   The method of claim 1 wherein said analyte comprises a glycoprotein, glycopeptide, or glycolipid.

6.   The method of claim 1 wherein said analyte
25 comprises an ion.

7.   The method of claim 1 wherein said analyte comprises a protein or peptide.

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8. The method of claim 7 wherein said protein or
peptide is selected from the group consisting essentially
of enzymes, hormones, lipoproteins, and antibodies.

9. The method of claim 1 wherein said analyte
5   comprises a steroid.

10. The method of claim 1 wherein said analyte
comprises an antigen or a hapten.

11. The method of claim 1 wherein said analyte
comprises theophylline.

10   12. The method of claim 1 wherein said analyte
comprises creatinin.

13. The method of claim 1 wherein said analyte
comprises a drug.

14. The method of claim 1 wherein said analyte
15   comprises a polynucleotide.

15. The method of claim 1 wherein said analyte
comprises a pesticide.

16. The method of claim 1 wherein said
fluorescence reagent comprises a fluorophore or compound
20   labelled with said fluorophore that binds directly to
said analyte.

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17. The method of claim 16 wherein said
fluorescence is measured by measuring the change in
fluorescence intensity of said fluorophore or compound
labelled with said fluorophore upon binding to said
5 analyte.

18. The method of claim 16 wherein said
fluorescence is measured by measuring the change in the
excitation or emission spectrum of said fluorophore or
compound labelled with said fluorophore upon binding to
10 said analyte.

19. The method of claim 18 wherein the change in
said excitation or emission spectrum of said fluorophore
or compound labelled with said fluorophore is measured by
measuring (a) the appearance or disappearance of emission
15 peaks, (b) the ratio of the signal observed at two or
more emission wavelengths, (c) the appearance or
disappearance of excitation peaks, or (d) the ratio of
the signal observed at two or more excitation
wavelengths.

20   20. The method of claim 16 wherein said
fluorescence is measured by measuring changes in the
excited state lifetime of said fluorophore or compound
labelled with said fluorophore.

21. The method of claim 1 wherein said
25 fluorescence reagent comprises an analogue of said
analyte labelled with a fluorophore and a ligand capable
of binding to both said analogue and said analyte.

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22.   The method of claim 21 wherein said
fluorescence is measured by measuring the change in
fluorescence intensity of said analogue labelled with
said fluorophore upon binding to said analyte.

5   23.   The method of claim 21 wherein said
fluorescence is measured by measuring the change in the
excitation or emission spectrum of said analogue labelled
with said fluorophore upon binding to said analyte.

24.   The method of claim 23 wherein the change in
10 said excitation or emission spectrum of said analogue
labelled with said fluorophore is measured by measuring
(a) the appearance or disappearance of emission peaks,
(b) the ratio of the signal observed at two or more
emission wavelengths, (c) the appearance or disappearance
15 of excitation peaks, or (d) the ratio of the signal
observed at two or more excitation wavelengths.

25.   The method of claim 21 wherein said
fluorescence is measured by measuring changes in the
excited state lifetime of said analogue labelled with
20 said fluorophore.

26.   The method of claim 1 wherein said
fluorescence reagent comprises an analogue of said
analyte and a ligand labelled with a fluorophore and
capable of binding to both said analogue and said
25 analyte.

27.   The method of claim 26 wherein said
fluorescence is measured by measuring the change in
fluorescence intensity of said ligand labelled with said
fluorophore upon binding to said analyte.

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28.   The method of claim 26 wherein said
fluorescence is measured by measuring the change in the
excitation or emission spectrum of said ligand labelled
with said fluorophore upon binding to said analyte.

5   29.   The method of claim 28 wherein the change in
said excitation or emission spectrum of said ligand
labelled with said fluorophore is measured by measuring
(a) the appearance or disappearance of emission peaks,
(b) the ratio of the signal observed at two or more
10 emission wavelengths, (c) the appearance or disappearance
of excitation peaks, or (d) the ratio of the signal
observed at two or more excitation wavelengths.

30.   The method of claim 26 wherein said
fluorescence is measured by measuring changes in the
15 excited state lifetime of said ligand labelled with said
fluorophore.

31.   The method of claim 21 wherein said ligand
is selected from the group consisting essentially of an
antibody, a receptor for said analyte, a lectin, an
20 enzyme, and a lipoprotein.

32.   The method of claim 1 wherein said
fluorescence reagent is labelled with an energy-absorbing
donor molecule and an energy-absorbing acceptor molecule,
the excited state energy level of the donor overlapping
25 with the excited state energy level of the acceptor, and
said fluorescence is measured by determining the extent
to which non-radiative fluorescence resonance energy
transfer occurs between the donor and the acceptor upon
binding.

33.   The method of claim 32 wherein said
fluorescence reagent comprises a specific binding pair,
one member of which is labelled with said donor molecule
and the other member of which is labelled with said
5 acceptor molecule.

34.   The method of claim 33 wherein said specific
binding pair comprises a ligand capable of binding to
said analyte and a analogue of said analyte capable of
binding to said ligand.

10   35.   The method of claim 32 wherein said
fluorescence reagent comprises (a) a first ligand
labelled with said energy-absorbing donor molecule and
capable of binding to a first portion of said analyte and
(b) a second ligand labelled with said energy-absorbing
15 acceptor molecule and capable of binding to a second
portion of said analyte different from said first
portion, and said fluorescence is measured by determining
the extent to which non-radiative fluorescence resonance
energy transfer occurs between the donor and the acceptor
20 upon binding.

36.   The method of claim 35 wherein said first and
second ligands are antibodies.

37.   The method of claim 35 wherein said first and
second ligands are oligonucleotides.

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38.   The method of claim 32 wherein said
fluorescence reagent comprises an agent capable of
binding to said analyte, said agent being labelled with
both said energy-absorbing donor molecule and said
5 energy-absorbing acceptor molecule, said fluorescence
being measured by determining the extent to which non-
radiative fluorescence resonance energy transfer occurs
between the donor and the acceptor upon binding.

39.   The method of claim 32 wherein said
10 illumination excites the donor and non-radiative
fluorescence resonance energy transfer is determined by
measuring the ratio of the fluorescence signal at two
emission wavelengths, one of which is due to donor
emission and the other of which is due to acceptor
15 emission.

40.   The method of claim 32 wherein said
illumination is chosen such that it excites the donor at
a first wavelength and the acceptor at a second
wavelength, and non-radiative fluorescence resonance
20 energy transfer is determined by measuring the ratio of
the fluorescence signal due to the acceptor following
donor excitation and the fluorescence signal due to the
acceptor following acceptor excitation.

41.   The method of claim 32 wherein said non-
25 radiative fluorescence resonance energy transfer is
determined by assessing whether there is a decrease in
donor lifetime, a quenching of donor fluorescence, or an
enhancement of acceptor fluorescence intensity.

42.   The method of claim 32 wherein one or both of
30 the donor-acceptor pair are fluorophores.

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43.   The method of claim 1 wherein said the
detection of said analyte comprises an irreversible
chemical reaction, with an accompanying change in
fluorescence.

5   44.   The method of claim 43 wherein said
fluorescence reagent comprises an enzyme and a co-factor
or substrate that reacts with said analyte in the
presence of said enzyme with an accompanying fluorescence
change.

10   45.   The method of claim 43 wherein said analyte
is an enzyme and said fluorescence reagent comprises a
substrate that is altered by said enzyme with an
accompanying fluorescence change.

46.   The method of claim 43 wherein said
15 fluorescence reagent comprises (a) an enzyme in whose
presence said analyte reacts to form a detectable product
and (b) an indicator for detecting the presence of said
product.

47.   The method of claim 43 wherein said
20 fluorescence reagent comprises (a) a substrate or co-
factor capable of reacting with an enzyme to cause a
detectable fluorescence change, (b) a first ligand
labelled with a first portion said enzyme and capable of
binding to a first portion of said analyte, and (c) a
25 second ligand labelled with a second portion of said
enzyme and capable of binding to a second portion of said
analyte different from said first portion,
neither said first portion of said enzyme nor said
second portion of said enzyme being capable individually
30 of interacting with said substrate or co-factor to
produce a detectable fluorescence change, -

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the binding of said first and second ligands to
said analyte causing said first and second portions of
said enzyme to interact with each other so as to
reconstitute said enzyme, thereby permitting reaction
5 with said substrate or co-factor.

48.   The method of claim 43 wherein said
fluorescence reagent comprises (a) a substrate or co-
factor capable of reacting with an enzyme to cause a
detectable fluorescence change, (b) a ligand labelled
10 with a first portion said enzyme and capable of binding
to said analyte, and (c) an analogue of said analyte
labelled with a second portion of said enzyme and capable
of binding to said ligand,
neither said first portion of said enzyme nor said
15 second portion of said enzyme being capable individually
of interacting with said substrate or co-factor to
produce a detectable fluorescence change,
the binding of said ligand and said analogue to
each other causing said first and second portions of said
20 enzyme to interact with each other so as to reconstitute
said enzyme, thereby permitting reaction with said
substrate or co-factor,
the presence of said analyte disrupting binding
between said analogue and said ligand.

25   49.   The method of claim 43 wherein said
fluorescence is measured by measuring the change in
fluorescence intensity of said fluorescence reagent
associated with the appearance of said detectable
product.

30   50.   The method of claim 43 wherein said
fluorescence is measured by measuring the change in
excitation or emission spectrum of said fluorescence

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reagent associated with the appearance of said detectable product.

51.   The method of claim 50 wherein the change in said excitation or emission spectrum is measured by
5 measuring (a) the appearance or disappearance of emission peaks, (b) the ratio of the signal observed at two emission wavelengths, (c) the appearance or disappearance of excitation peaks, or (d) the ratio of the signal observed at two excitation wavelengths.

10   52.   The method of claim 43 wherein said fluorescence is measured by measuring changes in the excited state lifetime of said fluorescence reagent associated with the appearance of said detectable product.

15   53.   The method of claim 1 wherein said sensor is provided with a selectively permeable membrane that permits analyte to diffuse in and out of said sensor but prevents said fluorescence reagent from diffusing out of said sensor.

20   54.   The method of claim 1 wherein at least some of the components of said fluorescence reagent are immobilized within said sensor.

55.   The method of claim 54 wherein said sensor comprises a porous matrix in which at least some of the
25 components of said fluorescence reagent are immobilized within the pores of said matrix.

56.   The method of claim 1 wherein at least some of the components of said fluorescence reagent are freely mobile within said sensor.

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57.   The method of claim 56 wherein the components
of said fluorescence reagent are admixed with an adjuvant
and injected as a bolus into said individual.

58.   The method of claim 1 further comprising
5 implanting in said individual a drug delivery system in
communication with said sensor,
said drug delivery system configured to release a
prescribed amount of a drug in response to the level of
analyte detected by said sensor

10   59.   An _in vivo_ method for detecting an analyte in
an individual comprising the steps of
a) placing a sensor in communication with the body
fluids of said individual suspected of containing said
analyte,
15   said sensor comprising a fluorescence reagent for
detecting said analyte that reversibly binds to said
analyte,
said sensor being configured to retain said
fluorescence reagent while allowing analyte to diffuse
20 into and out of said sensor,
said fluorescence reagent comprising an energy-
absorbing donor molecule and an energy-absorbing acceptor
molecule, the excited state energy level of the donor
overlapping with the excited state energy level of the
25 acceptor;
b) illuminating said sensor so as to
i) excite the donor or
ii) excite both the donor and acceptor; and
c) measuring the fluorescence from said
30 fluorescence reagent associated with the presence of said
analyte in said individual by determining the extent to
which non-radiative fluorescence resonance energy

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transfer occurs between the donor and the acceptor upon binding,

said non-radiative fluorescence resonance energy transfer being determined by measuring

5   i) the ratio of the fluorescence signal at two emission wavelengths, one of which is due to donor emission and the other of which is due to acceptor emission, when only the donor is excited, or

          ii) the ratio of the fluorescence signal due to
10 the acceptor following donor excitation and the fluorescence signal due to the acceptor following acceptor excitation.

          60.   An _in vivo_ sensor for non-invasively detecting an analyte in an individual comprising a
15 fluorescence reagent for detecting said analyte,

          the fluorescence signal from said fluorescence reagent being indicative of the presence of said analyte in the individual,

          said sensor being configured such that (a) once
20 implanted in the individual said sensor does not exit the skin of the individual, and (b) said fluorescence reagent is retained within said sensor while analyte is allowed to diffuse into and out of said sensor.

          61.   The method of claim 26 wherein said ligand is
25 selected from the group consisting essentially of an antibody, a receptor for said analyte, a lectin, an enzyme, and a lipoprotein.
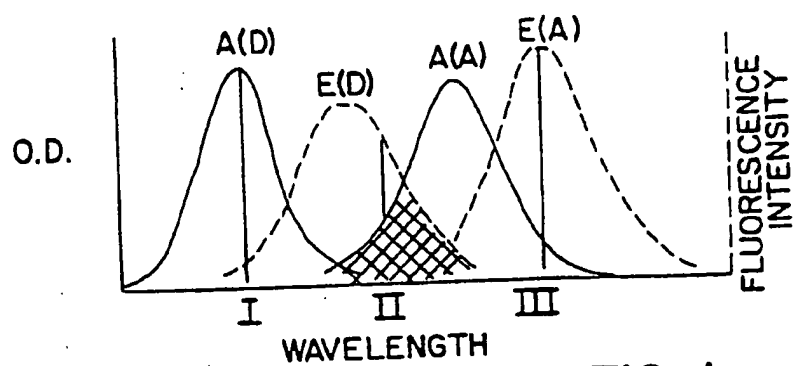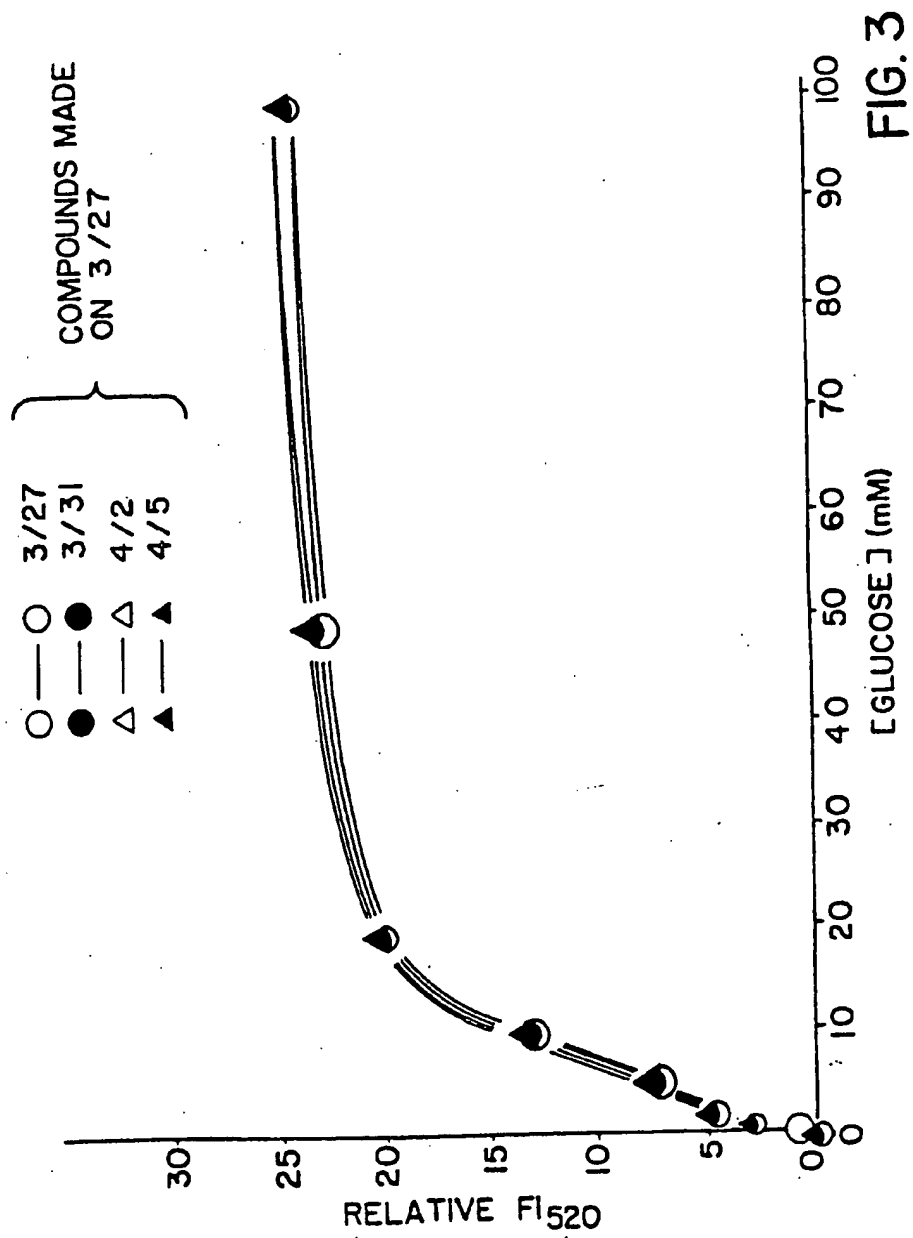
FIG. la
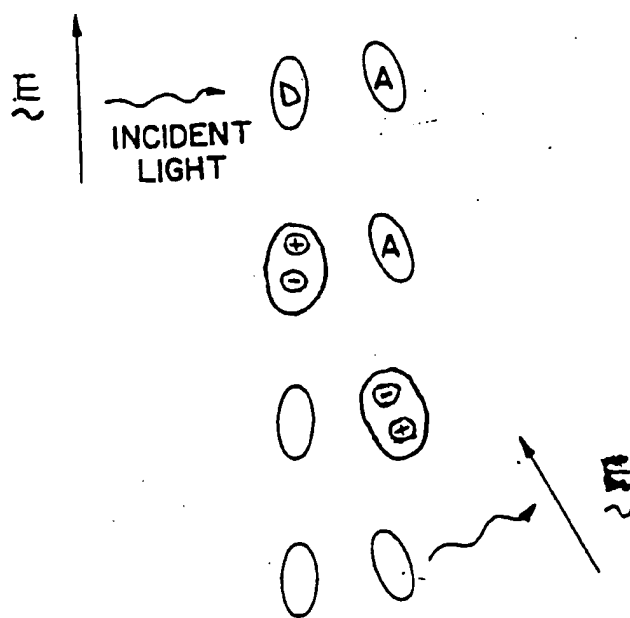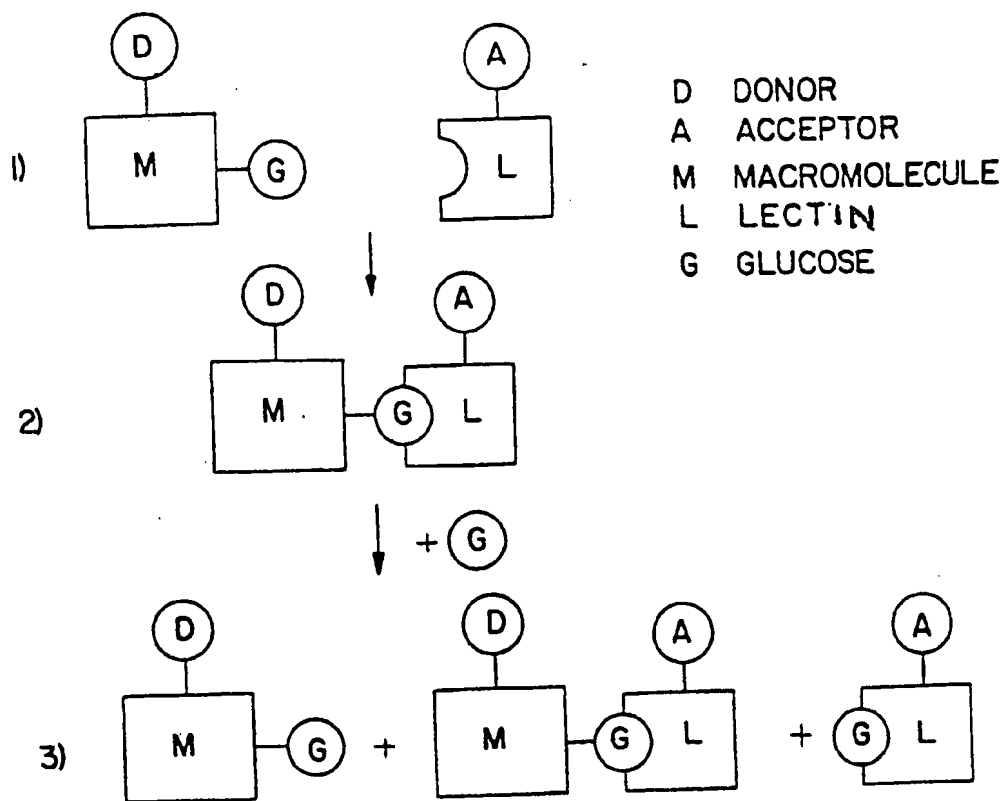
FIG. lb

FIG. 2

FIG. 3

FIG. 4

MIXTURE DIALYZED AGAINST DIFFERENT GLUCOSE CONCENTRATIONS

0 = 0 mM GLUCOSE IN HBSS
5 = 5 mM GLUCOSE ADDED TO HS
HS = HORSE SERUM
10 = 10 mM GLUCOSE ADDED TO HS

FIG. 5

FIG. 6b

(2)

RELATIVE FLUORESCENCE INTENSITY
(ARBITRARY UNITS)

FIG. 6a

(1)

RELATIVE FLUORESCENCE INTENSITY
(ARBITRARY UNITS)

FIG. 7

Stability study; 8 weeks.

FIG. 8

Stability study; Acrylic Fiber;

FIG. 9

FIG. 10

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FIG. 11

Fluorescent Group

Ligand

Binding of analyte
causes change in
fluorescence of ligand

A

Analyte

FIG. 12

Analyte has two
Binding sites

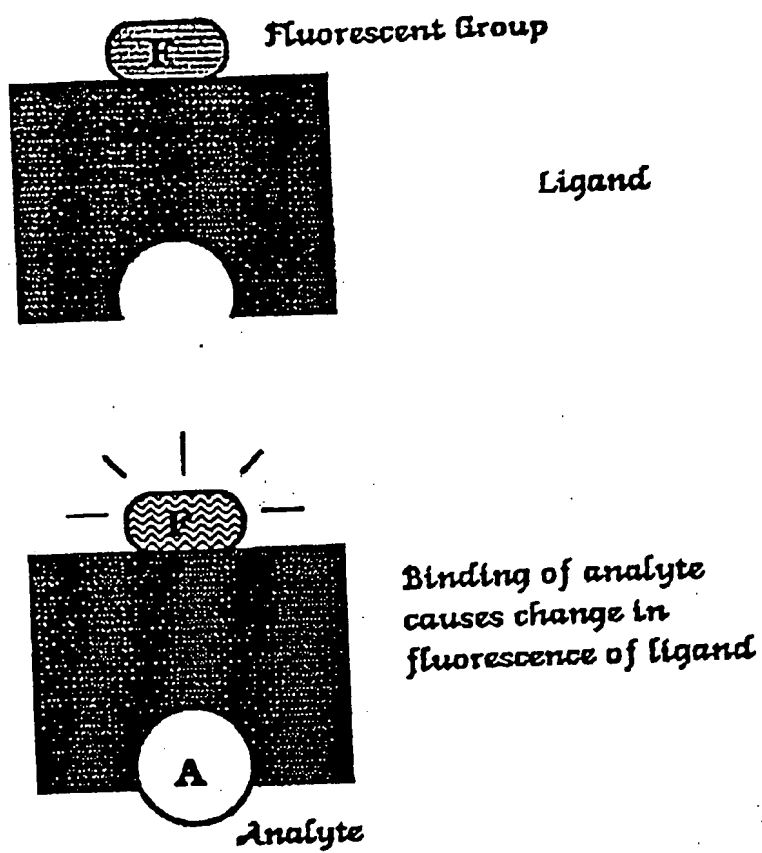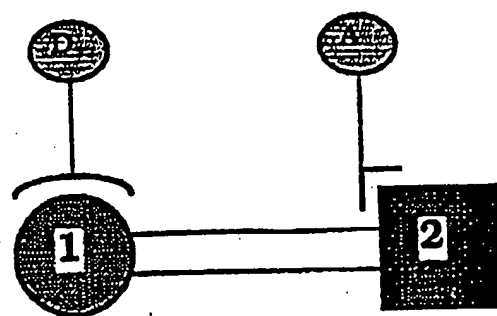Ligand for site 1
labelled with
donor

Ligand for site 2
labelled with
acceptor
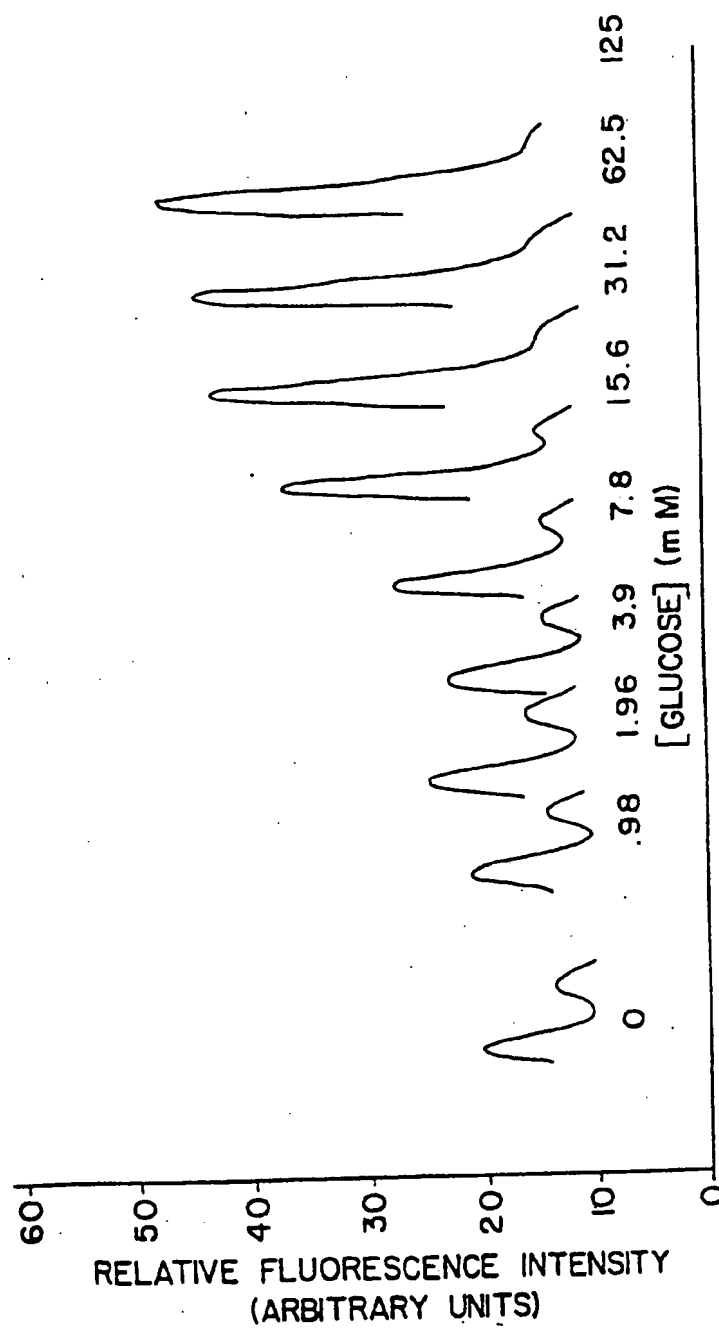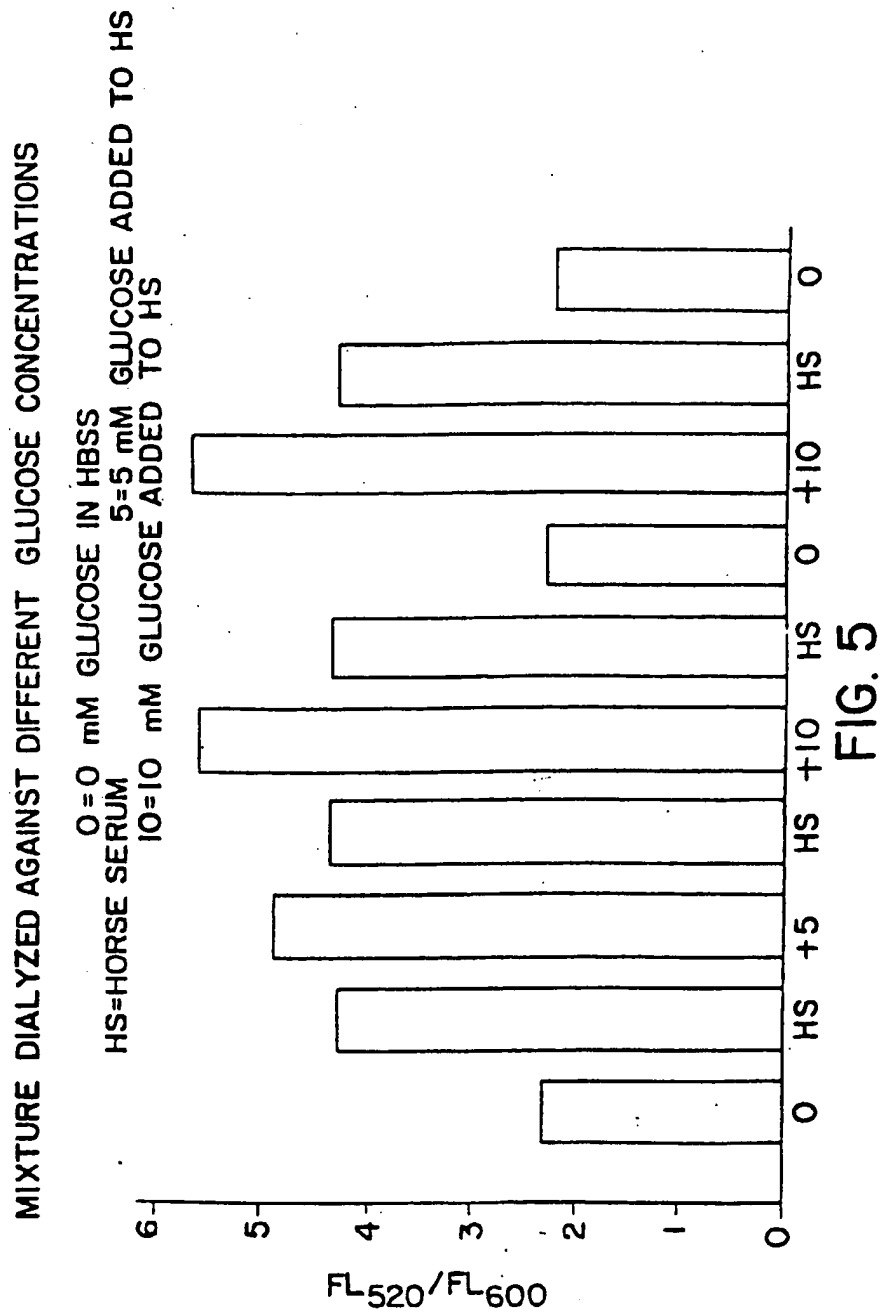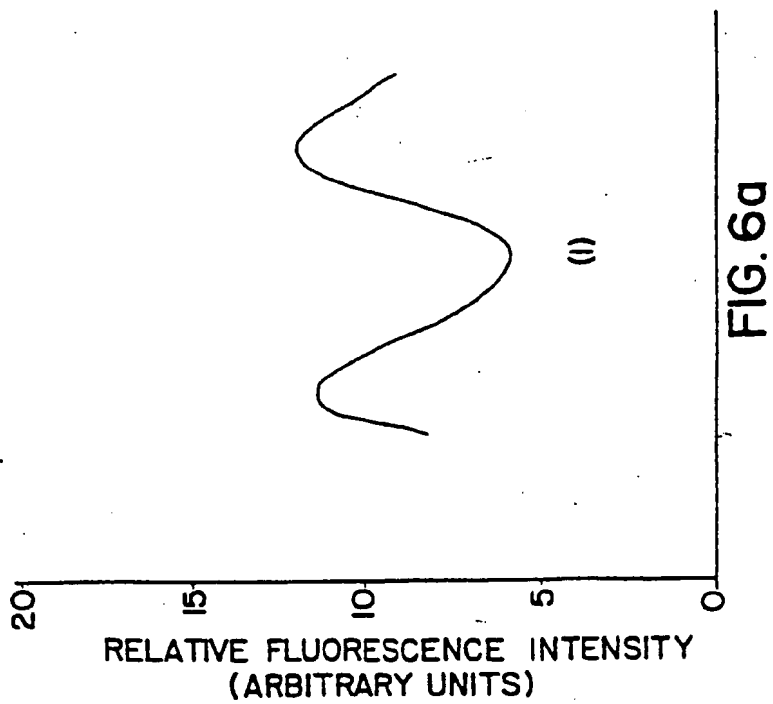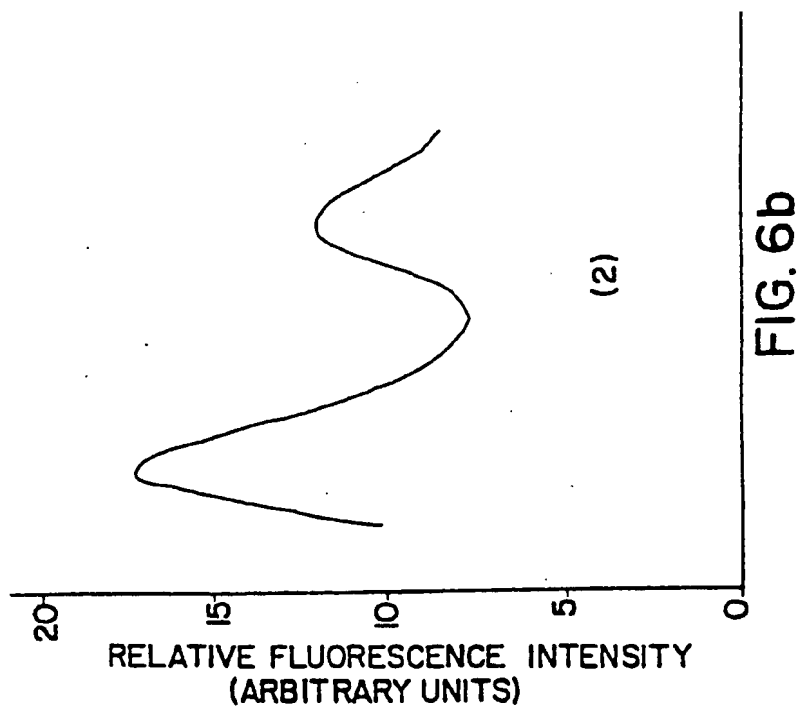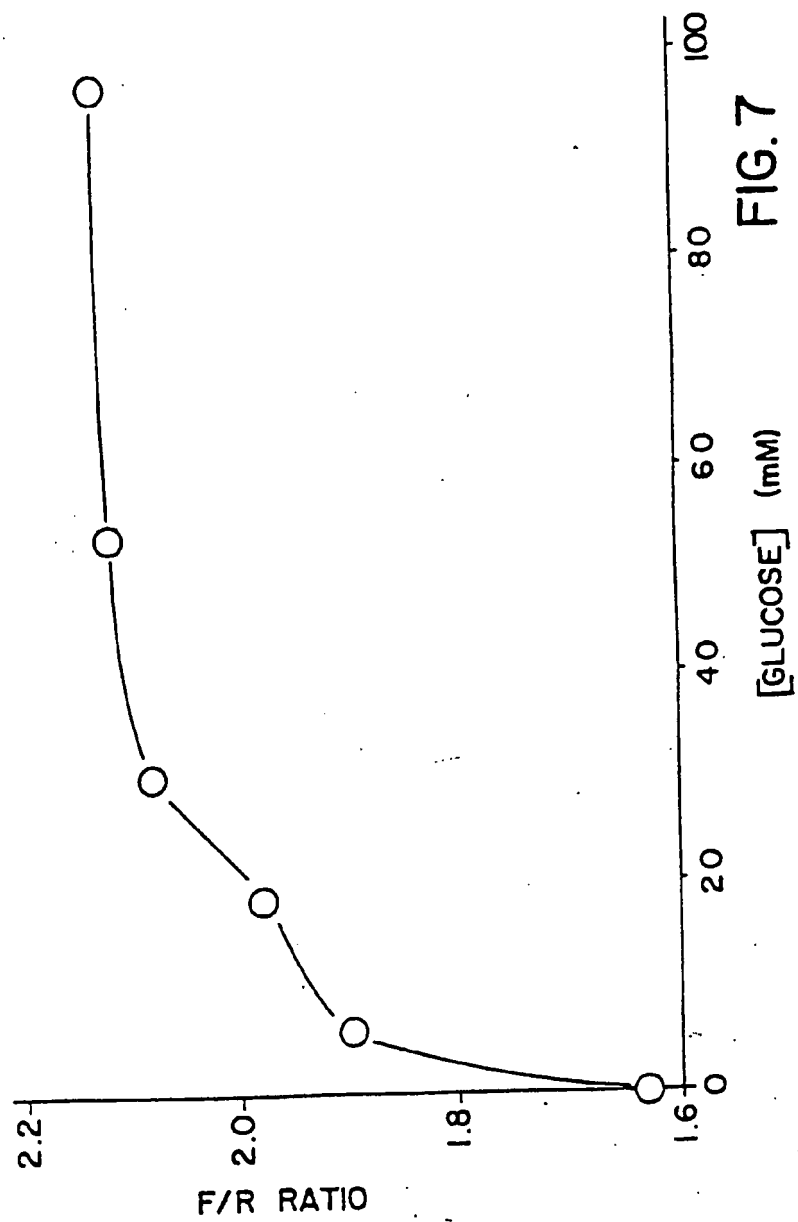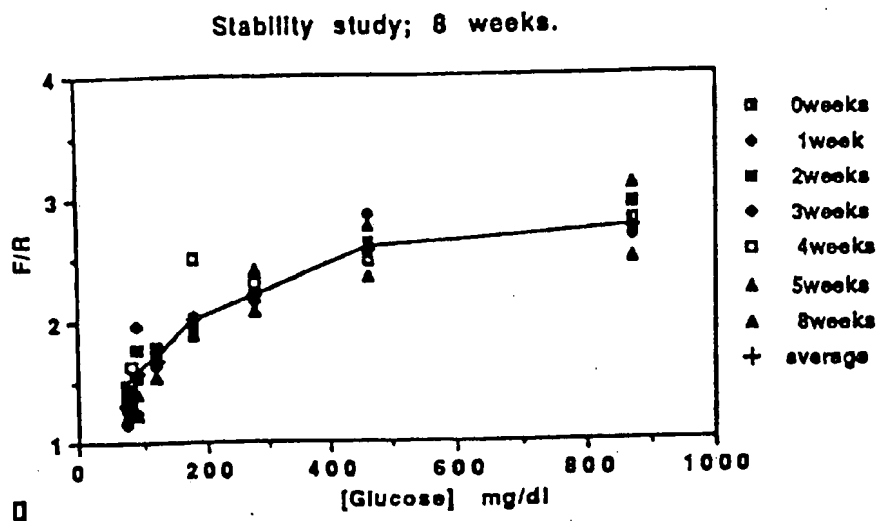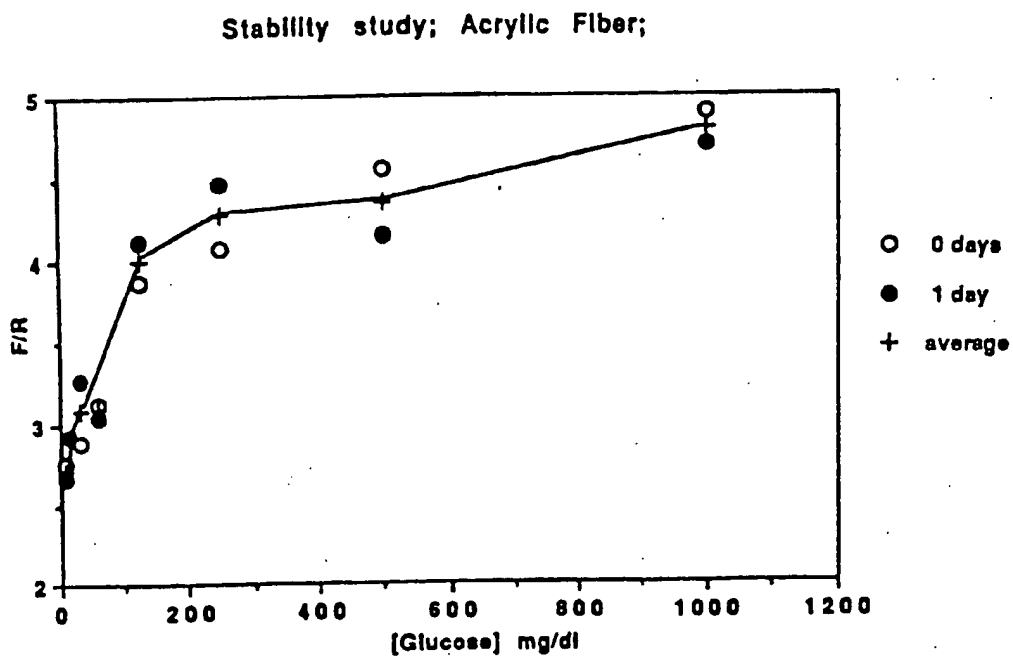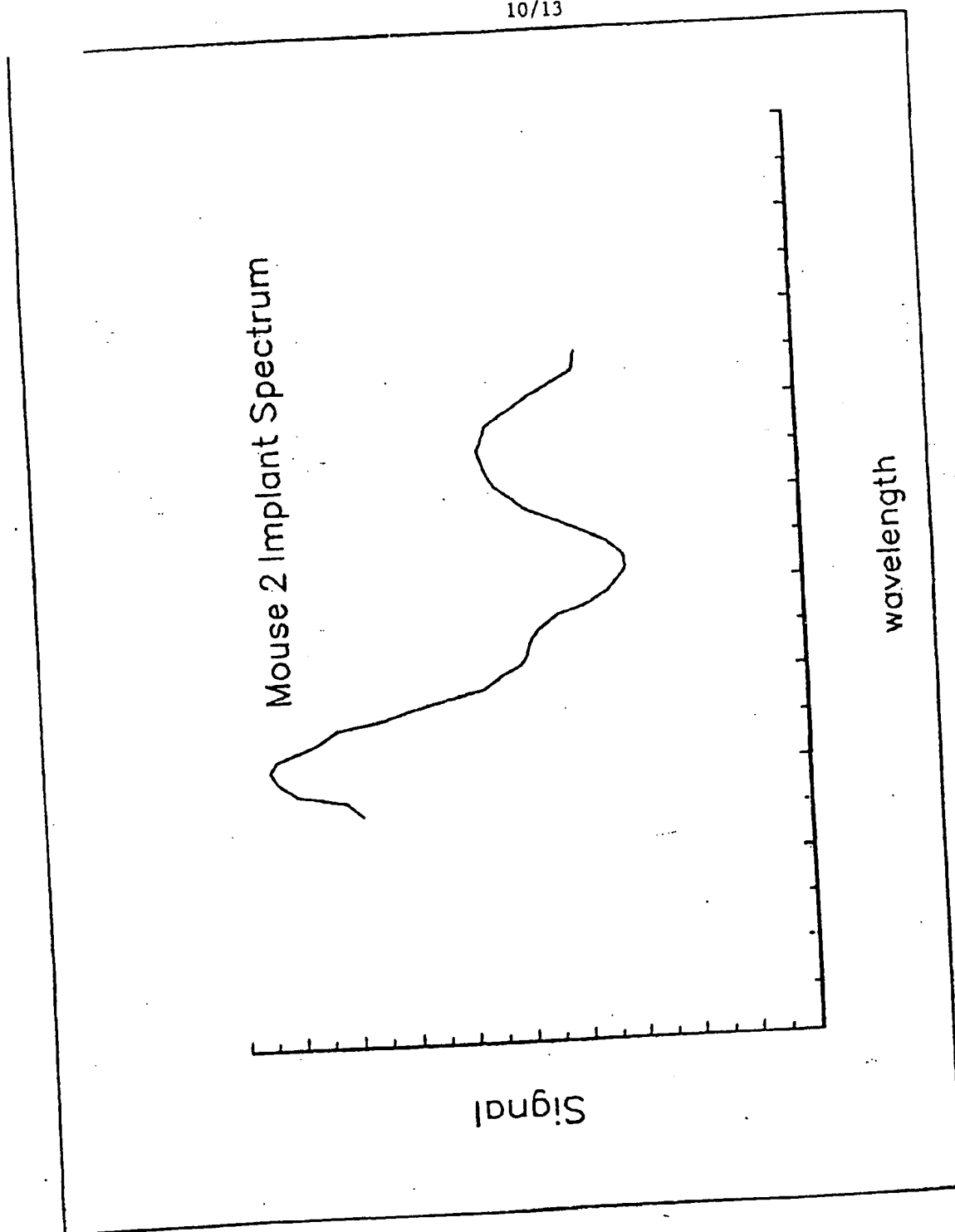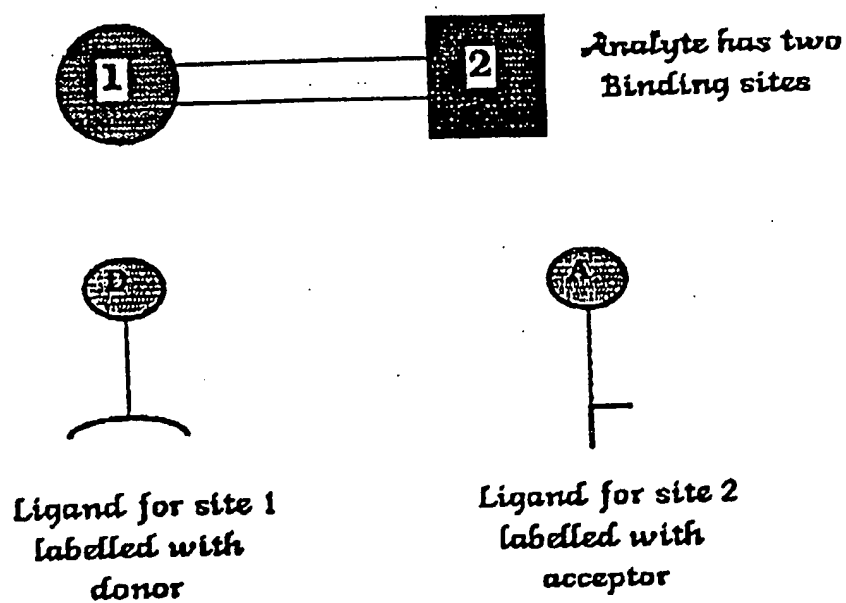
Concurrent binding of two ligands to analyte brings
donor and acceptor into proximity => FRET

FIG. 13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68
US CL :435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 4, 7.4, 7.9, 7.92, 7.72; 436/95, 501, 537, 805, 807, 815; 604/632, 633; 424/9; 128/634

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG, BIOCHM File (one search)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, UNITED STATES OF AMERICA, Volume 85, Number 23, issued December 1988, R.A. Cardullo, et al., "Detection of nucleic acid hybridization by nonradioactive fluorescence resonance energy transfer", pages 8790-8794, see entire document.	1-61
Y	TALANTA, Volume 35, Number 2, issued 1988, D. Meadows, et al., "Fiber-optic bisensors based on fluorescence energy transfer", pages 145-150, see entire document.	1-61
Y	RAPID DETECTION AND IDENTIFICATION OF INFECTIOUS AGENTS, issued 1985, M. J. Heller, et al., "Chemiluminscent and fluorescent probes for DNA hybridization systems" pages 245-256, see entire document.	1-61

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 August 1993	AUG 30 1993

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Margaret Parr
Telephone No. (703) 308-0196 |

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,330,299 (CERAMI) 18 MAY 1982, see entire document.	1-61
X,P Y,P	US, A, 5,143,066 (KOMIVES, et al.) 01 SEPTEMBER 1992, see entire document.	60 1-59,61
Y	US, A, 4,401,122 (CLARK, JR.) 30 AUGUST 1983, see entire document.	1-61